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<p>Cytoskeletal remodeling is crucial in many cellular events, including the adhesion, spreading and motility of breast cancer cells. The Rho family of small GTPases (Rho, Rac and Cdc42) are signal transducers that regulate cytoskeleton dynamics. Our studies have shown that Rac1 is important for cell spreading, a biological process in which the cytoskeleton is highly active. Rac activity is high only during early stages of spreading. The activity of p-21activating kinase (PAK), an effector molecule for Rac and Cdc42 GTPases, is also high only during the early stages of spreading. Overexpression of catalytically active PAK inhibits cell spreading and decreases myosin phosphorylation. Myosin is the cytoskeletal protein which provides the force generating ability to the actin cytoskeleton. We showed this effect of PAK was due to phosphorylation and inhibition of myosin light chain kinase (MLCK), the enzyme that phosphorylates the light chain of myosin II. We also found a second target of PAK to be LIM kinase, which regulates actin depolymerization.</p> <p style="text-align: right;">Our recent work has focused on the observation that PAKs are highly active in certain breast cancer cell lines. We have attempted to determine why PAK activity is elevated in these cells, whether downstream mediator activity is similarly increased, and how this contributes to overall breast cancer motility.</p>			
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Introduction

Tumor invasion and metastasis are the major cause of cancer mortality. This is particularly true for breast cancer since metastasis of the primary tumor leads to tumor inaccessibility and, consequently, greater mortality. Many studies have shown the importance of signaling molecules in changes that are associated with transformed phenotypes (1-3). In fact, many signaling molecules are affiliated with cytoskeleton changes that accompany the motile or metastatic phenotype. The Rho family of small GTPases (Rho, Rac and Cdc42) are members of the Ras superfamily; all of which regulate cell function via conversion between a GTP-bound active state and a GDP-bound inactive form. Recently, it has become clear that the Rho family mediates morphological and cytoskeletal changes of both normal and transformed cells (4,5). Rho activation leads to stress fiber formation and focal adhesions. Activation of Rac leads to membrane ruffles and lamellipodia formation. Similarly, Cdc42 regulates the extension of actin filament bundles into filopodia. The mechanisms by which the Rho family of GTPases regulate cytoskeleton remodeling is not well understood.

Dynamic rearrangement of the cytoskeleton is, in part, driven by actin polymerization and actin-myosin interactions. Myosins are mechanoenzymes which generate force along actin filaments and, thus, are crucial for cell movements, including cytokinesis, pseudopod formation, polarized growth and cell migration (6-8). Changes in the expression of myosin isoforms have been linked to the transformed phenotype in both melanoma and breast cancers (9-11). Recently, Rho has been shown to regulate myosin activity through Rho kinase, an effector molecule for Rho (12,13). Rho kinase phosphorylates myosin phosphatase and inhibits its function, consequently acting to increase myosin phosphorylation. Therefore, Rho GTPase seems, in part, to regulate cytoskeleton changes by activating Rho kinase and thereby increasing myosin phosphorylation and effecting its force generating abilities.

In this grant we proposed to examine the effects of p21 activated kinase (PAK), an effector molecule for Rac and Cdc42, on myosin phosphorylation during migration of breast cancer cells (Aims 2 & 3). Our results in the previous annual report indicated that PAK acts to inhibit myosin phosphorylation, the opposite effect that Rho kinase had on myosin. This inhibition is due to PAK phosphorylation and inhibition of myosin light chain kinase (MLCK), a kinase known to phosphorylate myosin and thereby regulate its activity (14). We also reported that PAK acted on LIM kinase to regulate actin assembly by inhibiting depolymerization (15).

PAK 1 and PAK2 activity has been reported to be elevated in certain breast cancer cell lines (16). We verified this in two lines, ZR75 cells and SKBR3 cells (Fig.1). Our studies have been directed at trying to understand the mechanism by which such abnormal PAK regulation occurs and the consequences of elevated PAK activity for breast cancer cell motility.

RESULTS [Note: This final report reflects the contributions of Dr. Luraynne Sanders up until 04-20-01, and the subsequent work of Dr. Elizabeth Jeanclos from that date until the end of the grant on 07-31-01]

We observed that elevated PAK activity in the SKBR3 and ZR75 cells was associated with a poorly motile phenotype in which the cells had extremely large focal adhesions and abundant, thickened stress fibers (Fig.2). While reminiscent of a Rho-induced phenotype, we could detect no increases in Rho activity as determined by direct measurement using a Rhotekin Rho-binding domain pulldown assay (data not shown). Similarly, no elevation in Rac or Cdc42 activity was detected with a p21-activated kinase p21-binding domain (PBD) assay (17). The latter data indicated that elevated PAK activity was not due to increased GTPase stimulation, and this was confirmed by

the inability of the Rho GTPase-inactivating toxin B from *C. difficile* to decrease PAK activity.

Examination of PAK subcellular localization led to the surprising observation that active PAK exclusively localized to focal adhesions (Fig.3). Since this mislocalization was not Rho GTPase-dependent, we considered two possible mechanisms that have been proposed to link PAK to focal adhesions: the binding of the adapter protein Nck to the first pro-rich domain in PAK (18) and/or the binding of PAK via its 4th pro-rich domain to PIX, a guanine nucleotide exchange factor (19). Both PIX and Nck were found to be present in the enlarged focal adhesions (not shown). To address this question, we used the approach of microinjecting peptides encompassing the Nck and PIX binding sites on PAK, with the rationale that these would specifically disrupt binding of each protein to PAK in vivo. Indeed, we observed that the PIX-disrupting peptide aa. 147-231 effectively caused dissociation of PAK from focal adhesions, while the NCK-selective peptide had no effect (Fig.4). We conclude that PAK is mislocalized to focal adhesions by recruitment via PIX.

Disruption of focal adhesions by either making cells non-adherent or by treatment with the cytoskeleton-disrupting agent cytochalasin d was associated with a loss of PAK activity. These data suggest that PAK becomes activated because of its association with PIX in the focal adhesion. We are attempting to verify this hypothesis.

Conclusion

Cell adhesion, migration and invasion play a critical role in understanding tumor metastasis. Comprehending cytoskeleton dynamics is pivotal in understanding the complexities of metastasis. Thus, our studies initially focused on the process of spreading in order to understand what is occurring at the moving edge of the cell. At present we are working on Aim 3 in our proposal, which is to examine the importance of PAK-myosin interactions on breast cancer cell migration. With the development of the SFV viral gene expression system we were able to transfect breast cancer cells with

high enough transfection efficiency to do migration assays in Boyden chambers (Technical Objective 3: task 5 and 6). In this report we provide evidence that the constitutively active Rac/Cdc42 effector PAK is associated with a poorly motile breast cancer cell phenotype consisting of enlarged focal adhesion assemblies. PAK is mislocalized and, likely, activated due to a PIX-induced localization to focal adhesion structures

PAK Kinase Activity and PAK Levels in Breast Cancer Cell Lines

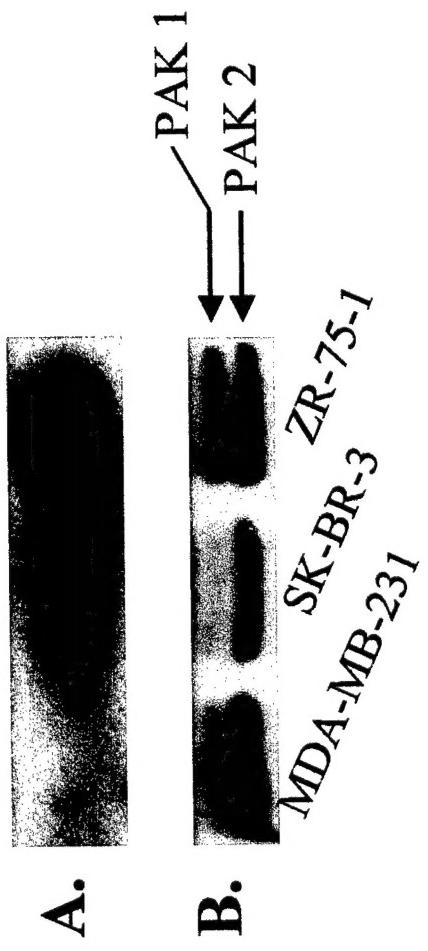


Figure 1: **A.)** PAK kinase activity in MDA-MB-231, SK-BR-3 and ZR-75-1 breast cancer cell lines was measured by a kinase assay. Note the elevated PAK kinase activity in the SK-BR-3 and ZR-75-1 cells as compared to the MDA-MB-231 cells. Equivalent amounts of protein were analyzed. **B.)** Whole cell lysates from the indicated cell lines were analyzed for PAK protein expression levels. ZR-75-1 cells express equivalent amounts of PAK1 and PAK2, while the MDA-MB-231 and SK-BR-3 cells express mostly PAK2.

Breast Cancer Cells with Activated PAK have Larger Actin Filaments and Focal Adhesions

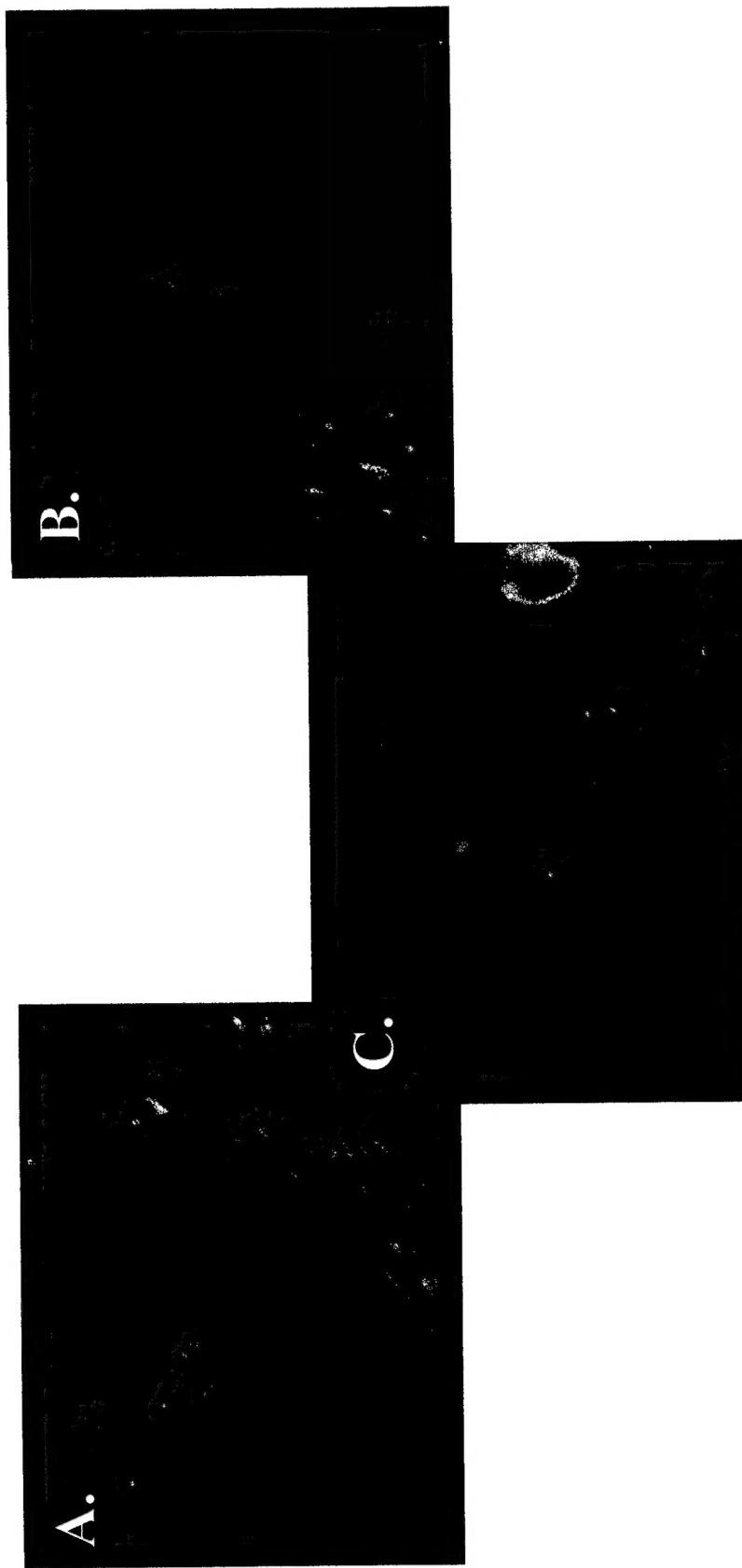


Figure 2: SK-BR-3, ZR-75-1, and MDA-MB-231 breast cancer cells were stained with phalloidin (red) to reveal the actin cytoskeleton and anti-vinculin (green) to visualize the focal adhesions. The merge indicates localization of vinculin-containing focal adhesions at the ends of actin filaments. **A)** SK-BR-3 and **B)** ZR-75-1 cells, breast cancer cells with high PAK activity, have much larger focal adhesions and actin filaments than **C)** MDA-MB-231 breast cancer cells with low PAK activity.

Breast Cancer Cells with High PAK Activity Mislocalize PAK to Focal Adhesions

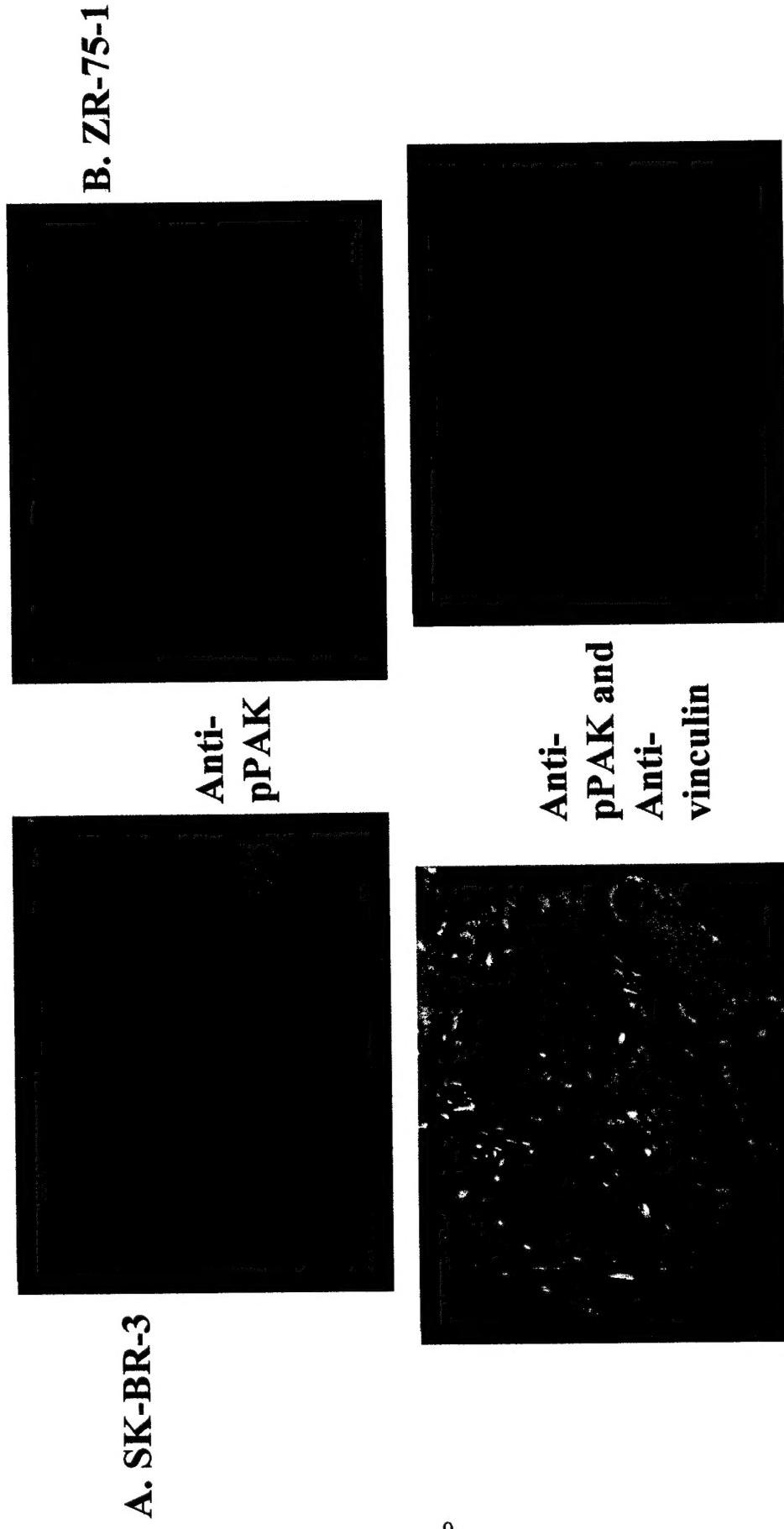


Figure 3: A) SK-BR-3 cells (60x magnification) and B) ZR-75-1 cells (100x magnification) were stained with an antibody that recognizes activated PAK (anti-pPAK in green, top panels), and co-stained with anti-vinculin (red, bottom panels) to visualize focal adhesions. The merge (orange) demonstrates active PAK localizes to these structures. The MDA-MB-231 breast cancer cells which have low PAK activity do not show localization of Pak to focal adhesions (data not shown).

The PIX Binding Domain of PAK Displaces Overexpressed PAK from Focal Adhesions

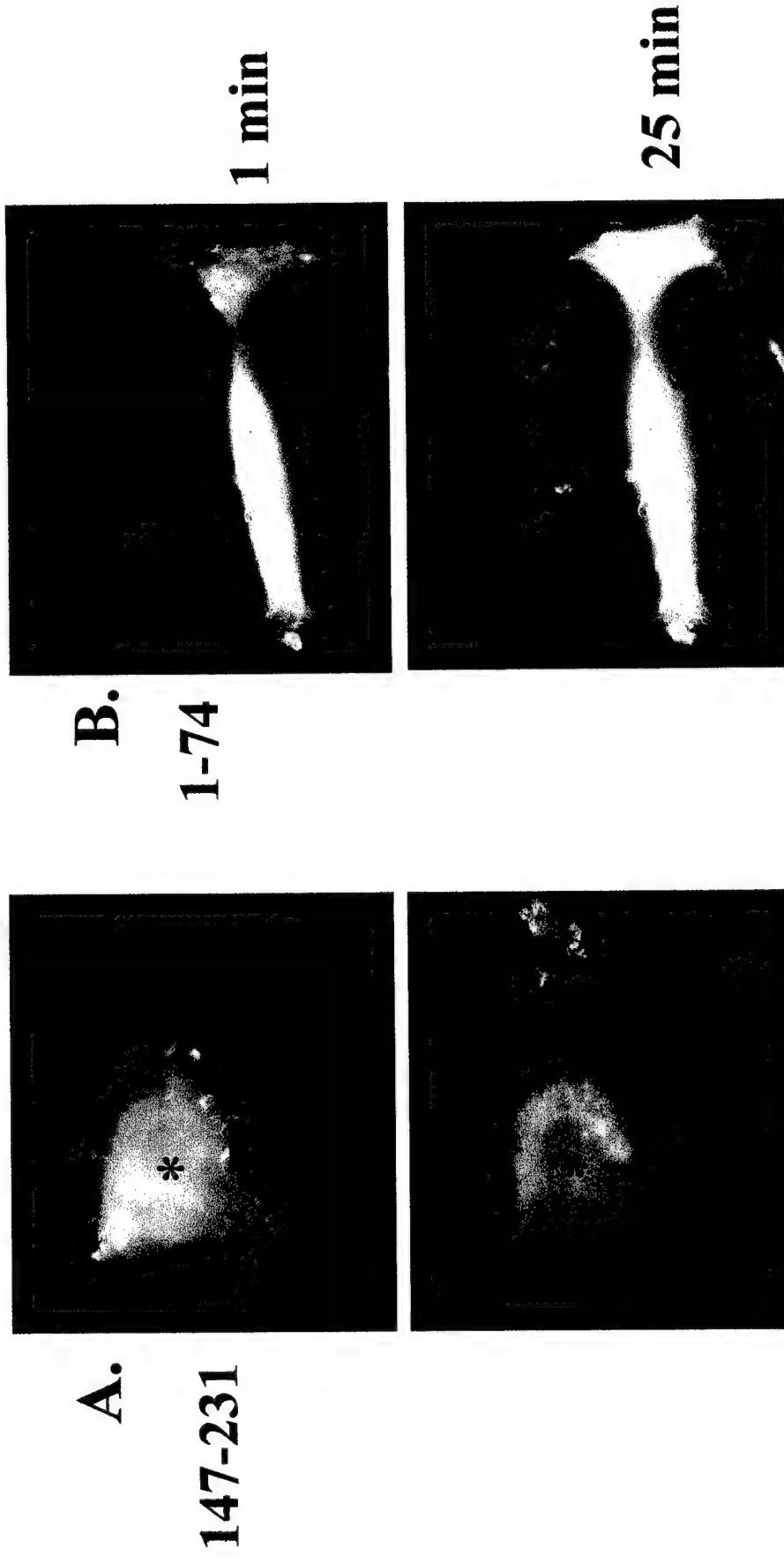


Figure 4: SK-BR-3 cells were transfected with a green fluorescent protein (GFP)-tagged PAK construct which localizes to focal adhesions. **A)** Cells labelled with an asterisk were microinjected with a peptide encoding amino acids 147-231 of PAK which disrupts PAK/PIX interaction. After 25 min, there was a decrease in the amount of GFP- PAK in focal adhesions as compared with the non-injected cell. **B)** Cells with an asterisk were microinjected with a peptide encoding amino acids 1-74 of PAK, which does not displace GFP-PAK from focal adhesions.

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